PATENT
2503-1170

IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of

Daniele CALISTRI et al. Conf. 1643

Application No. 10/547,669 Group 1637

Filed September 2, 2005 Examiner M. Staples

METHOD FOR THE IDENTIFICATION OF COLORECTAL TUMORS

DECLARATION UNDER RULE 132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Daniele Calistri, hereby declare as follows:

My relevant background and experience are set forth on the attached c.v. I make this declaration in support of the present application, and to provide evidence in rebuttal of several contentions set forth in the outstanding Official Action.

I do not believe that any of the publications cited in the Office Action mailed September 14, 2007, taken alone or in combination, disclose or suggest the claimed invention. In particular, I do not believe that any of the publications disclose or suggest the primers recited in the claims.

Table 1 shows the results, in terms of FL-DNA (Fluorescence long DNA) values expressed as nanograms (see also patent description), obtained by two different approaches:

i) a method that utilizes the primers recited in

the claimed invention (method 1), and

ii) a method that utilizes a series of primers designed in the same genomic regions (method 2).

For the FL-DNA analysis, the same set of specimens were used in both methods: stool samples from 10 different colorectal cancer patients (disease confirmed by endoscopic examination and histological evaluation of the tissue) and 9 healthy donors without a history or symptoms of colorectal cancer (confirmed by colonoscopy). DNA extraction from stool was performed once for each sample and the same DNA was utilized for the DNA integrity analysis (FL-DNA) in the two methods to avoid bias due to the different amounts and/or quality of genomic DNA.

Moreover, to compare the amplification results obtained from the two methods and to avoid other biases the same standard curve (same genomic DNA as reference, with same dilutions), same conditions of electrophoresis and fluorescence analysis were used.

The two methods differ only for the primers, and obviously for the amplification conditions, which are strictly related to the primers to obtain the best possible results for each amplicon.

The results show that the two sets of data are not comparable in many cases, indicating that the use of a selected series of primers determine different FL-DNA values. This difference is also indicative of a different ability to discriminate between cancer patients and healthy subjects. In fact, the aim of the FL-DNA method is to

identify cancer patients with the highest sensitivity and specificity possible.

Tables 2a/b show the sensitivity and specificity of the two different approaches. In Table 2a, using the primers cited in our claims, it is possible to obtain good sensitivity and specificity with different cut-offs (see, for example, 10-15 or 20 ng cut-offs). Conversely, using other primers in the same genomic region as the claimed primers (Table 2b), sensitivity and specificity are rarely high for the same cut-off, and good sensitivity tends to correlate with poor specificity, and vice-versa. This problem does not allow one to identify an accurate (high sensitivity and specificity) cut-off for colorectal cancer detection.

Table 1

Results of FL-DNA analysis using different primers

Tumor no.	FL-DNA (ng) "Claimed" primers (method 1)	FL-DNA (ng) "New" primers (method 2)
1	34	33
2	41	9
3	19	12
4	42	12
5	10	10
6	9	3
7	35	3
8	21	8
9	96	6
10	77	11
Healthy donor no.		
1	9	6
2	13	9
3	13	7
4	14	13
6	4	13
6	15	2
7	25	12
8	17	4
9	0	2

Table 2a

Sensitivity and specificity of FL-DNA analysis with "claimed" primers (method 1)

Cut-off (ng)	Sensitivity no. cases	%	Specificity no. cases	%
10	9	90	7	30
15	8	80	3	70
20	7	70	2	80
25	6	60	2	80
30	6	60	1	90

Table 2b

Sensitivity and specificity of FL-DNA analysis with "new" primers (method 2)

Cut-off (ng)	Sensitivity no. cases	%	Specificity no. cases	%
5	8	80	6	40
10	5	50	3	70
15	1	10	0	100
20	1	10	0	100
25	1	10	0	100

Methods

DNA extraction (common to the two methods)

Genomic DNA was extracted from 10-20 mg of feces obtained from the spiral groove of the commercially available immunochemical Fecal Occult Blood Test (FOBT (iFOBT OC-Sensor, Alfa Wassermann).

1) One milliliter of TE buffer is added to a 1.5-ml test tube and used to wash the stool attached to the spiral groove.
2) Centrifuge for 15 minutes at 5,000 g. Transfer the supernatant to a clean sterile test tube and add 155 μl of ammonium acetate 7.5 M and 930 μl of ethanol 100%. Mix and centrifuge for 15 minutes at 5,000 g.
3) Extract DNA from the pellet using QIAmp DNA stool kit.
4) Add 50 μl of H_2O, 100 μl of ammonium acetate 4 mol/L and 200 μl of isopropanol 100%. Mix all and incubate at -20°C. for 20 minutes. Centrifuge at 14,000 g, wash pellet with 200 μl of ethanol 70%. Dry pellet at 37°C for 30 minutes and resuspend in 40 μl of H_2O.

Amplification and FL-DNA analysis is performed in the same way as described in the patent application, the final result is normalized to the data obtained from 4 gr of stool multiplying the results by 3. This value was obtained by evaluating the results of FL-DNA obtained from 4 gr and 10-20 mg of stool in the same samples. A similar normalization value could be obtained using the same approach and quantities other than the standard 4 gr.

Fluorescence long DNA (FL-DNA) analysis

Method 1 The FL-DNA analysis using the primers listed in the Table at page 2, par. [0028] of US2006/0216713, was performed as previously described in the patent application.

Method 2 (analysis with new primers): For this

analysis we designed a new set of primers, obtaining the amplification of 8 new fragments from the same chromosomal regions used for method 1.

From the p53 gene sequences:

exon 5: 5A(forward): FAM-CAA CTC TGT CTC CTT CCT CTT CC

5B(reverse): AAC CAG CCC TGT CGT CTC T

exon 6: 6A(Forward): CAG GCC TCT GAT TCC TCA CT

6B(reverse): HEX-CTT AAC CCC TCC TCC CAG AG

exon 7: 7A(forward): FAM-TCA TCT TGG GCC TGT GTT ATC

7B(reverse) TGG AAG AAA TCG GTA AGA GGT G

exon 8: 8A(forward) GGG ACA GGT AGG ACC TGA TTT

8B(reverse) HEX-TAA CTG CAC CCT TGG TCT CC.

Form the APC gene sequences:

fragment 1: 1A(forward)CCC TAG AAC CAA ATC CAG CA

1B(reverse) HEX-CAT TC ACT GCA TGG TTC AC

fragment 2: 2A(forward) FAM- GTG AAC CAT GCA GTG GAA TG

2B(reverse)CAC TCA GGC TGG ATG AAC AA;

fragment 3: 3A(Forward)AAG AAG CTC TGC TGC CCA TA

3B(reverse): HEX- GTG AAC CAT GCA GTG GAA TG

fragment 4: 4A(forward) FAM- GTC AAT ACC CAG CCG ACC TA

4B(reverse) GTC AAT ACC CAG CCG ACC TA.

The p53 exons 5 to 8 and fragments 1 to 4 of APC were amplified in a final volume of 25 ml containing 2 μl of stool DNA, 0.4 mM of each primer, 200 mM of deoxynucleotide (Takara Bio Inc), 1X reaction buffer with 3.5 mM MgCl2 (Qiagen), and 1 U of Taq polymerase (Qiagen). The reaction mixture was subjected to 32 cycles: 60 s at 94°C and then 60 s at 58°C f followed by incubation at 72°C for 60 s. for all

fragments, Primers used were end-labeled with fluorochromes provided by Applied Bioystems.

For both methods DNA from each sample was quantified on a standard curve of genomic DNA (1, 2, 5, 10 and 20 ng) normalized to 100, and expressed as nanograms.

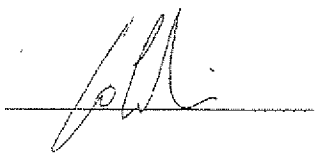
For both methods we analyzed DNA fragment intensity by electrophoresis using a 3100 Avant Genetic Analyzer (Applied Biosystems) equipped with GeneScan Analysis 3.7. The final FL-DNA value was obtained by analyzing the fluorescence intensity of each sample-specific PCR product. The quantification of each sample was calculated by reference to a standard curve (1, 2, 5, 10, and 20 ng) of genomic DNA and expressed as nanograms.

The DNA samples were obtained from a small amount of feces (10-15 mg) and for this reason the FL-DNA value obtained using the claimed primers was normalized to the data obtained from 4 gr of stool multiplying the results by 3.

Upon reviewing the results, it is apparent that not all primers are equivalent or perform in the same way, as suggested by the Official Action. This is probably due to the DNA used for the analysis. The paradigm that different primers located in the same region determine similar results is true mainly if we consider DNA extracted under optimal conditions and if genomic DNA of high molecular weight is obtained. FL-DNA analysis was not performed in this way. It must be understood that the genomic DNA extracted from stool is not in the best

conditions and is more extensively degraded than genomic DNA extracted from blood or tissues. This is probably a consequence of the status of the starting material and, as also suggested in the patent, is related to the presence/absence of colorectal cancer cells. For these reasons, primers cannot be considered equivalent to each other because even slight differences in primer sequences could determine a difference in amplification efficiency, resulting in a more or less accurate evaluation of DNA integrity and consequently in a better or poorer capacity to identify colorectal cancer patients.

The undersigned declare further that all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.



19/01/2009
Date